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Heparin, Heparan Sulfate, and Dermatan Sulfate Regulate Formation of the Insulin-like Growth Factor-I and Insulin-like Growth Factor-binding Protein Complexes*

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The mechanisms by which insulin-like growth factor-I (IGF-I) is released from insulin-like growth factor binding proteins (IGFBPs) and then binds to its receptor have not been defined. This study was designed to determine the role of glycosaminoglycans in altering the formation of the IGF-I·IGFBP complexes. Heparin inhibited formation of the IGF-I·IGFBP-5 complex and also separated preformed IGF-I·IGFBP-5 complexes. Heparin also inhibited formation of the IGF-I·IGFBP-3 complex; however, it did not inhibit formation of complexes between IGF-I and IGFBP-1, -2, or -4. Heparin exposure was associated with a 17-fold decrease in the affinity of IGFBP-5 for IGF-I. A synthetic peptide that contains residues from Arg^{221} to Arg^{238} of IGFBP-5, and a heparin binding domain prevented the inhibitory effects of heparin on formation of the IGF-I·IGFBP-5 complex. It did not directly compete with IGF-I for binding to IGFBP-5, suggesting that heparin binding to this region of IGFBP-5 resulted in a conformational change in IGFBP-5 which lowered its affinity for IGF-I. Other glycosaminoglycans that contained *O*-linked sulfates in the 2 or 3 carbon positions of iduronic acid, *e.g.* heparan sulfate and dermatan sulfate, also inhibited the IGF-I·IGFBP-5 complex formation, whereas those that did not, such as keratan sulfate or hyaluronic acid, had minimal effects. Anionic polysaccharides that contained *O*-sulfate groups in the 2 or 3 positions, such as dextran sulfate, pentosan polysulfate, and fucoidan, also had inhibitory activity. The findings suggest a role for these compounds in inhibiting IGF-I·IGFBP interactions, thus making IGF-I available to bind to its receptor.

Insulin-like growth factor I (IGF-I)[1] cellular actions have been shown to be exerted through the binding of IGF-I to its receptor (1); however, most of IGF-I in extracellular fluids is bound to insulin-like growth factor binding proteins (IGFBPs). The IGFBPs have been shown to stimulate or to inhibit IGF-I-mediated cellular actions (2–4). Binding of IGF-I to high affinity forms of IGFBPs in conditioned medium prevents it from binding to its receptor, thereby inhibiting IGF-I mediated cellular actions (4, 5). On the other hand, the binding of IGF-I to cell surface or extracellular matrix (ECM) associated IGFBPs has been associated with potentiation of IGF-I's cellular actions (3, 5–7). In both situations it remains uncertain how IGF-I is released from IGFBPs in extracellular fluids or on cell surfaces in order to be able to bind to its receptor, an event that is usually required for optimal biologic responses.

Recently glycosaminoglycans (GAGs) and proteoglycans have been shown to modulate growth factor-stimulated cellular actions (8, 9). Basic fibroblast growth factor (b-FGF) binds to bovine capillary endothelial cell surface heparin-like molecules, which act as a reservoir of b-FGF and modulate the metabolism of b-FGF by cells (10). The biological half-life of acidic FGF is prolonged by heparin, thereby enhancing its cellular actions (11). Transforming growth factor β stimulates synthesis of decorin (9) and when transforming growth factor β is bound to decorin its biological activity is decreased (12).

Heparin binding sites in many proteins have been characterized and shown to contain multiple basic amino acids. Two motifs *X*-B-B-*X*-B-*X* and *X*-B-B-B-*X*-*X*-B-*X* (B: basic amino acid, *X*: nonbasic amino acid) have been proposed as consensus sequences for heparin binding. All of the forms of IGFBPs except for IGFBP-4 have at lease one of these putative heparin binding sequences (13, 14), suggesting that they have the potential to bind to heparin. Previous studies have shown that heparin can interfere with the formation of the IGF-I·IGFBP-3 complex (15, 16); therefore, we wondered whether heparin might affect the binding of IGF-I to other forms of IGFBPs. We report here that heparin inhibited the IGF-I·IGFBP-5 complex formation but had no effect on binding to IGFBP-1, -2, or -4. We have determined some of the structural requirements of heparin that are necessary for its inhibitory activity and have defined a region of IGFBP-5 that mediates heparin-induced inhibition of IGF-I binding.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGFBP-3, IGFBP-4, and IGFBP-5 were synthesized in transfected Chinese hamster ovary cells and purified as described previously (17). Recombinant human IGFBP-1 (5) and bovine IGFBP-2 (18) were prepared as published previously. Recombinant IGF-I was obtained from Bachem, Inc. (Torrance, CA). ^{125}I-IGF-I was a gift from Dr. Louis E. Underwood (University of North Carolina, Chapel Hill). ^{125}I-IGFBP-5 was prepared by a modified chloramine-T method (19). Three peptides that contain sequences of IGFBP-5 were synthesized and purified as described (20). They were designated peptide A (residues R^{221}-K-G-F-Y-K-R-K-Q-C-K-P-S-R-G-R-K-R^{238}), peptide B (residues A^{151}-V-K-K-D-R-R-K-K-L-T^{161}), and peptide D (residues ^{134}P-K-I-F-R-P-K-H-T-R-I-S-E-L-K-A-E^{150}), respectively. Heparin (187 USP units/mg) was purchased from Sigma. Heparin, heparan sulfate, chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, hyaluronic acid, and keratan sulfate were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Chemically modified heparins, including completely desulfated, *N*-acetylated heparin (CDSNAc-heparin), completely desulfated, *N*-resulfated heparin (CDSNS-heparin), and *N*-desulfated, *N*-

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[1] The abbreviations used are: IGF, insulin like-growth factor; IGFBPs, insulin-like growth factor binding proteins; GAG, glycosaminoglycan; FGF, fibroblast growth factor; DSS, disuccinimidyl suberate; EMEM, Eagle's minimum essential medium; PAGE, polyacrylamide gel electrophoresis; ECM, extracellular matrix; NDSNAc, *N*-desulfated *N*-acetylated heparin; CDSNAc, completely desulfated *N*-acetylated heparin; CDSNS, completely desulfated *N*-resulfated heparin.

acetylated heparin (NDSNAc-heparin) were purchased from Seikagaku Kogyo Co. Dextran sulfate (M_r: <10,000), pentosan polysulfate, and fucoidan were purchased from Sigma. Dithiothreitol was purchased from Sigma. Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co. Dimethyl sulfoxide was purchased from Mallinckrodt Chemical Co. (Paris, KY). Eagle's minimum essential medium (EMEM) was purchased from Hazelton (Denver, PA).

Affinity Cross-linking Studies—Cross-linking was performed according to a modification methods described previously (21, 22). ^{125}I-IGF-I or ^{125}I-IGFBP-5 (30,000 cpm/tube) was added to 100 µl of EMEM supplemented with 20 mM HEPES, pH 7.3, and was incubated at room temperature with IGFBP-1, -2, -3, -4, or -5 (100 ng/ml) or unlabeled IGF-I (100 ng/ml). Additional incubations were conducted using ^{125}I-IGF-I and heparan sulfate, chondroitin sulfate A or C, dermatan sulfate, keratan sulfate, or hyaluronic acid using 200 µg/ml. Chemically modified heparins, dextran sulfate, pentosan polysulfate, and fucoidan were also tested at 100 or 200 µg/ml. The synthetic peptides containing IGFBP-5 sequences termed peptides A, B, or D were tested at concentrations between 0.27 and 27 µM. After a 1-h incubation the samples were cross-linked by the addition of 10 µl of 5 mM DSS, which had been dissolved in dimethyl sulfoxide, to give a final concentration of 0.5 mM DSS for 20 min. The reaction was stopped by adding 10 µl of 0.5 M Tris-HCl, pH 7.4. Samples for SDS-PAGE were exposed to dithiothreitol in a final concentration of 0.1 M and Laemmli sample buffer (23). They were electrophoresed through a 12.5% gel. The gels were fixed with 25% isopropanol containing 10% acetic acid and 2.5% glycerol for 30 min then dried and autoradiographed using Kodak X-Omat film. In some experiments the autoradiographic intensities of radiolabeled bands were determined by scanning densitometry using a Hoffer scanning densitometer, model GS-300.

^{125}IGF-I Binding Assay—To determine the affinity of the IGFBPs for IGF-I, ^{125}I-IGF-I (20,000 CPM/tube) was incubated with 8 ng/ml of each form of IGFBP in 0.25 ml of 0.05 M HEPES, 0.1% bovine serum albumin, pH 6.0. Duplicate tubes received increasing concentrations of unlabeled IGF-I (0.4–10 ng/ml) and some tubes also received heparin 10 µg/ml. The bound and free IGF-I were separated by precipitation using 12.5% polyethylene glycol (M_r 8000–12,000) (Sigma) as described previously (5). The data were analyzed according to the method of Scatchard.

Binding of IGFBPs to Heparin—To determine if each form of IGFBP could bind to heparin, 4 µg/ml of each was incubated with heparin sepharose beads (5 µl) (Pharmacia Biotech Inc.) in EMEM, supplemented with 20 mM HEPES and 0.02% Tween 20, pH 7.3, in a final volume of 50 µl. To correct for nonspecific binding duplicate tubes containing an equal amount of IGFBP, and Sepharose beads but no heparin were included. After an overnight incubation at 4 °C, the samples were centrifuged at 16,000 × *g* for 1 min. The supernatants (24 µl) were electrophoresed using a 12.5% SDS gel then ligand blotted and autoradiographed. The pellets were washed with 200 µl of the same buffer then incubated for 10 min at 60 °C in 35 µl of Laemmli sample buffer then centrifuged. The IGFBPs in these supernatants were also analyzed by ligand blotting.

RESULTS

Heparin was shown to inhibit formation of the ^{125}I-IGF-I·IGFBP-5 complex (Fig. 1*A*). ^{125}I-IGF-I migrated to the bottom of the gel, whereas when it was incubated with IGFBP-5, a band with an approximate molecular mass of 47 kDa was detected. Labeling of this band could be inhibited if unlabeled IGF-I was added, indicating that the cross-linking of ^{125}I-IGF-I to IGFBP-5 was specific. Increasing concentrations of heparin reduced intensity of the 47-kDa band, indicating that heparin inhibited ^{125}I-IGF-I·IGFBP-5 complex formation. Scanning densitometry showed that the band intensity was reduced 56% with 1.0 µg/ml heparin and 83% with 100 µg/ml. In order to confirm the results, ^{125}I-IGFBP-5 was incubated without (*lane 1*) or with (*lanes 2–7*) 100 ng/ml of IGF-I in the absence (*lanes 1, 2,* and *7*) or presence (*lanes 3–6*) of the indicated concentrations of heparin (Fig. 1*B*). When ^{125}I-IGFBP-5 was incubated without IGF-I, two bands with molecular mass estimates of 39 and 23 kDa were detected. When ^{125}I-IGFBP-5 was incubated with IGF-I, a 47-kDa band was detected in addition to the molecular mass 39 kDa and the 23-kDa bands. However, in the presence of heparin the intensity of the 47-kDa band was decreased, and the intensity of the 39-kDa band was increased,

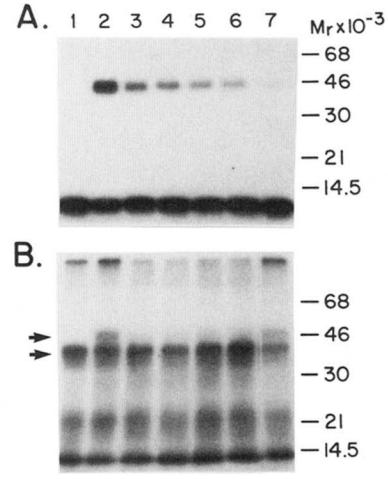

FIG. 1. *A*, inhibitory effects of heparin on forming the ^{125}I-IGF-I·IGFBP-5 complex. ^{125}I-IGF-I (30,000 cpm/tube) was added to 100 µl of EMEM supplemented with 20 mM HEPES, pH 7.3. The mixture was incubated with 100 ng/ml of IGFBP-5 and the indicated concentrations of heparin at room temperature. After a 1-h incubation, samples were cross-linked by adding DSS in a final concentration of 0.5 mM for 20 min and then the reaction was stopped by addition of 10 µl of 0.5 M Tris-HCl, pH 7.4. Samples for SDS-PAGE were prepared in a final concentration of 0.1 M of dithiothreitol and in Laemmli sample buffer, then electrophoresed through a 12.5% gel. The gel was fixed with 25% isopropanol containing 10% acetic acid and 2.5% glycerol, dried, and autoradiographed. *Lane 1*, no IGFBP-5; *lanes 2–7*, IGFBP-5, 100 ng/ml; *lane 3*, heparin, 1.0 µg/ml; *lane 4*, heparin, 2.5 µg/ml; *lane 5*, heparin, 10 µg/ml; *lane 6*, heparin, 100 µg/ml; *lane 7*, unlabeled IGF-I, 100 ng/ml. *B*, inhibitory effects of heparin on formation of the ^{125}I-IGFBP-5·IGF-I complex. ^{125}I-IGFBP-5 (30,000 cpm/tube) was added to 100 µl of EMEM supplemented with 20 mM HEPES, pH 7.3, and incubated with 100 ng/ml of IGF-I and the indicated concentrations of heparin at room temperature. After a 1-h incubation, samples were cross-linked using DSS, electrophoresed, and autoradiographed as described in the legend to Fig. 1*A*. The *upper arrow* represents the ^{125}I-IGF-I·IGFBP-5 complex, and the *lower arrow* represents ^{125}I-IGFBP-5. *Lane 1*, no IGF-I; *lanes 2–7*, IGF-I, 100 ng/ml; *lane 3*, heparin, 1 µg/ml; *lane 4*, heparin, 2.5 µg/ml; *lane 5*, heparin, 10 µg/ml; *lane 6*, heparin, 100 µg/ml; *lane 7*, unlabeled IGFBP-5, 100 ng/ml.

indicating that heparin inhibited formation of the IGF-I·IGFBP-5 complex. The molecular weight estimate of IGFBP-5 was larger than that previously reported using nonreduced gels (15). When the molecular mass of ^{125}I-IGFBP-5 was determined under reducing and nonreducing conditions, molecular mass estimates of 39 and 31 kDa, respectively, were obtained (data not shown). The 31-kDa estimate agrees with that obtained previously using nonreducing conditions (5).

To determine the specificity of the effect of heparin on IGF-I complex formation with IGFBP-5, the effect of heparin on the cross-linking of ^{125}I-IGF-I to IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-4 was examined. ^{125}I-IGF-I was incubated with 100 ng/ml of each form of IGFBP in the presence or absence of 100 µg/ml of heparin. When the IGFBPs were incubated with ^{125}I-IGF-I in the absence of heparin, the IGF-I·IGFBP complexes were detected (Fig. 2). When heparin was added, the intensity of the ^{125}I-IGF-I·IGFBP-3 complex band was decreased (43% reduction by scanning densitometry) but the intensities of the ^{125}I-IGF-I·IGFBP-1, -2, or -4 complex bands were not changed, *e.g.* 107, 112 and 102% of control, respectively. If the heparin concentration was increased to 500 µg/ml, a 57% reduction in ^{125}I-IGFBP-3 band intensity was noted, but there was no change in the intensity of the IGF-I·IGFBP-4 complex (data not shown). To determine if these differences were due to the ability of each of the IGFBPs to bind to heparin, each form of IGFBP was incubated with heparin-Sepharose beads, and the amount of IGFBP bound to heparin was determined by SDS-PAGE followed by ligand blotting. As shown in Fig. 3 heparin-Sepha-

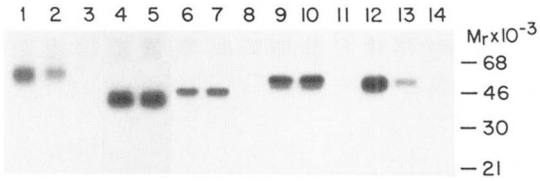

FIG. 2. **Inhibitory effects of heparin on the binding of the IGF-I to other forms of IGFBPs.** ^{125}I-IGF-I (30,000 cpm/tube) was incubated with 100 ng/ml of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 in the presence or absence of 100 μg/ml of heparin at room temperature in 100 μl of EMEM supplemented 20 mM HEPES, pH 7.3. After 1 h the samples were cross-linked using DSS as described previously, then SDS-PAGE was performed under reducing conditions, and the products were analyzed by autoradiography. *Lanes 1–3*, IGFBP-3; *lanes 4* and *5*, IGFBP-4; *lanes 6–8*, IGFBP-1; *lanes 9–11*, IGFBP-2; *lanes 12–14*, IGFBP-5. *Lanes 2, 5, 7, 10*, and *13*, heparin, 100 μg/ml. *Lanes 3, 8, 11*, and *14*, unlabeled IGF-I, 100 ng/ml.

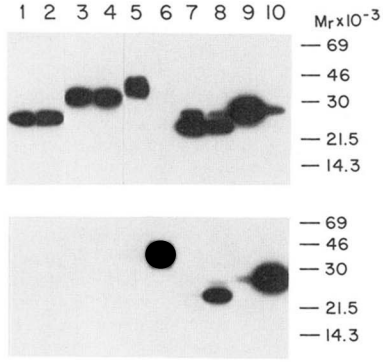

FIG. 3. **Binding of IGFBPs to heparin.** Each form of IGFBP (4 μg/ml) was incubated with heparin-Sepharose beads (5 μl) as described under "Experimental Procedures." The mixture was centrifuged, and the bound IGFBPs were released from the beads by heating to 60 °C for 10 min. in Laemmli sample buffer. The released proteins were separated by SDS-PAGE, transferred to Immobilon filters, and ligand-blotted. The *upper panel* shows the IGFBPs remaining in the supernatants, whereas the *lower panel* shows the amount of each IGFBP that was bound to sepharose (*lanes 1, 3, 5, 7*, and *9*) or heparin-Sepharose (*lanes 2, 4, 6, 8*, and *10*). *Lanes 1* and *2*, IGFBP-1; *lanes 3* and *4*, IGFBP-2; *lanes 5* and *6*, IGFBP-3; *lanes 7* and *8*, IGFBP-4; *lanes 9* and *10*, IGFBP-5.

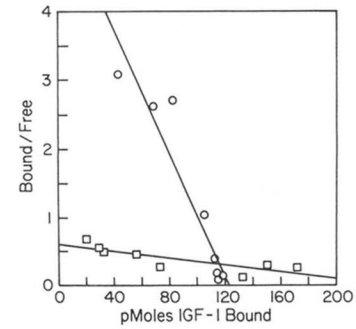

FIG. 4. **Scatchard plot of the effect of heparin on the binding of IGF-I to IGFBP-5.** ^{125}I-IGF-I (20,000 cpm) and IGFBP-5 (8 ng/ml) and increasing concentrations of unlabeled IGF-I were incubated in the presence (□) or absence (○) of 10 μg/ml heparin overnight at 4 °C as described under "Experimental Procedures." Bound and free IGF-I were separated by precipitation with 12.5% polyethylene glycol and centrifugation. The data are plotted using the method of Scatchard.

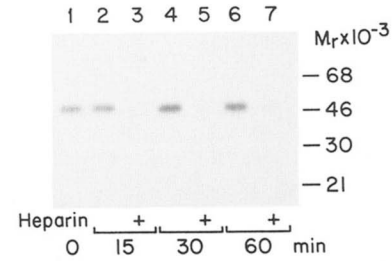

FIG. 5. **Time course of heparin-induced separation of the IGF-I·IGFBP-5 complex.** ^{125}I-IGF-I (30,000 cpm/tube) was added to 100 μl of EMEM supplemented with 20 mM HEPES, pH 7.3, and incubated with 100 ng/ml of IGFBP-5 at room temperature. After a 1-h incubation, selected samples were exposed to heparin, 100 μg/ml (*lanes 3, 5*, and *7*) (*lanes 2, 4, and 6*, no heparin) and then incubated for another 15 min (*lanes 2* and *3*), 30 min (*lanes 4* and *5*), and 60 min (*lanes 6* and *7*), respectively. At the indicated times, the samples were cross-linked and then electrophoresed and autoradiographed as described in the legend to Fig. 1*A*.

rose bound IGFBP-3, -4, and -5 but, not IGFBP-1 or -2. Since IGFBP-3, -4, and -5 bound heparin, but heparin inhibited the cross-linking of IGF-I to only IGFBP-3 and -5, we wished to determine the mechanism by which heparin was inhibiting IGF-I binding. Increasing concentrations of unlabeled IGF-I were incubated with ^{125}I-IGF-I and IGFBP-5 (8.0 ng/ml) in the presence or absence of heparin, and the bound IGF-I was precipitated using polyethylene glycol. Scatchard analysis of the binding data showed that coincubation with heparin (10 μg/ml) decreased the affinity of IGFBP-5 for IGF-I by 17-fold (Fig. 4). In contrast 10 μg/ml of heparin had no effect on the affinity of IGFBP-4 for IGF-I and resulted in only a 2-fold reduction in the affinity of IGFBP-3 (data not shown).

Since most of IGF-I is bound to IGFBPs in extracellular fluids, it is important to clarify whether heparin separates the IGF-I·IGFBP-5 complexes that have already formed. ^{125}I-IGF-I was incubated with 100 ng/ml of IGFBP-5 at room temperature (Fig. 5). After a 1-h incubation, the samples were exposed to 100 μg/ml of heparin (*lanes 3, 5*, and *7*) or heparin was omitted (*lanes 2, 4*, and *6*). The samples were then incubated for an additional 15 min (*lanes 2* and *3*), 30 min (*lanes 4* and *5*), or 60 min (*lanes 6* and *7*). After the first 1-h incubation, the IGF-I·IGFBP-5 complex was formed. Subsequent incubation with heparin separated this complex, since band intensities were decreased by 86, 91, and 93%, respectively, compared with that in the paired control sample without heparin. The separation of the IGF-I·IGFBP-5 complex by heparin was nearly complete by 15 min.

The effects of other GAGs on the inhibition of IGF-I·IGFBP-5 complex formation were examined. The intensity of the 47-kDa band in the samples was decreased by heparin, heparan sulfate, or dermatan sulfate compared with control (Fig. 6). The experiment was repeated seven times and the mean reductions in band intensities compared with control were heparin, 84.7 ± 6.6% ($p < 0.001$); heparan sulfate 78.2 ± 8.8% ($p < 0.001$); dermatan sulfate, 63.2 ± 20.1% ($p < 0.01$). In contrast the other glycosaminoglycans that were tested had minimal effects on the intensity of the ^{125}I-IGF-I·IGFBP-5 complex band. They were hyaluronic acid, 26.5 ± 11% (p, not significant); keratan sulfate, 7.4 ± 12.9% (p, not significant); chondroitin sulfate A, 17.2 ± 19% (p, not significant); and chondroitin sulfate C, 6.7 ± 9.4% (p, not significant).

Heparin has been shown to bind to several proteins, such as lipoproteins, growth factors, proteases, and protease inhibitors (8, 13). Heparin-binding proteins usually possess similar heparin-binding amino acid sequence motifs, including *X*-B-B-*X*-B-*X* and/or *X*-B-B-B-*X*-*X*-B-*X* (B: basic amino acid, X: nonbasic amino acid) (13). IGFBPs except for IGFBP-4 possess at least one of these putative heparin-binding amino acid motifs (14). IGFBP-5 and IGFBP-3 have two putative heparin-binding amino acid sequences: residues I^{175}-K-K-G-H-A^{180} and Y^{246}-K-K-K-Q-C-R-P^{253} in IGFBP-3 and residues P^{139}-K-H-T-R-I^{144} and Y^{225}-K-R-K-Q-C-K-P^{232}, in IGFBP-5. Therefore, we prepared a synthetic peptide (termed peptide A) containing the residues R^{221}-K-G-F-Y-K-R-K-Q-C-K-P-S-R-G-R-K-R^{238} to determine

FIG. 6. **Differences in the inhibitory effects of various glycosaminoglycans on formation of the IGF-I·IGFBP-5 complex.** ^{125}I-IGF-I (30,000 cpm/tube) was incubated with 100 ng/ml IGFBP-5 and 200 μg/ml of each glycosaminoglycan at room temperature. After a 1-h incubation, the samples were cross-linked, electrophoresed, and autoradiographed as described in the legend to Fig. 1*A*. *Lane 1*, no GAG; *lane 2*, heparin; *lane 3*, heparan sulfate; *lane 4*, hyaluronic acid; *lane 5*, keratan sulfate; *lane 6*, chondroitin sulfate A; *lane 7*, dermatan sulfate; *lane 8*, chondroitin sulfate C; *lane 9*, unlabeled IGF-I, 100 ng/ml.

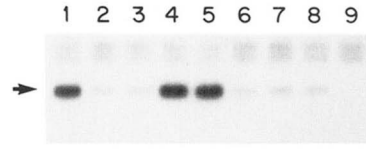

FIG. 7. **Reversal of the heparin effect with an IGFBP-5 peptide containing the heparin binding domain.** ^{125}I-IGF-I (30,000 cpm/tube) and 100 ng/ml IGFBP-5 (3.2 nM) were incubated with 10 μg/ml heparin in the presence or absence of peptide A or peptide B at room temperature for 1 h. The samples were cross-linked and subjected to SDS-PAGE as described in the legend to Fig. 1*A*. *Lanes 2–8*, heparin, 10 μg/ml. *Lanes 3, 4*, and *5*, peptide A, 0.27, 2.7, and 27 μM, respectively; *lanes 6, 7*, and *8*, peptide B, 0.27, 2.7, and 27 μM, respectively. *Lane 9*, unlabeled IGF-I, 100 ng/ml.

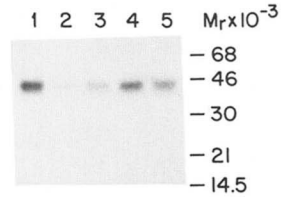

FIG. 8. **The effects of modified forms of heparin on formation of the IGF-I·IGFBP-5 complex.** ^{125}I-IGF-I (30,000 cpm/tube) in 100 μl of EMEM supplemented with 20 mM HEPES, pH 7.3, was incubated with 100 ng/ml IGFBP-5 and 200 μg/ml of modified heparins at room temperature for 1 h, then the samples were cross-linked and electrophoresed as in Fig. 1*A*. *Lane 1*, no heparin; *lane 2*, heparin; *lane 3*, ND-SNAc-heparin; *lane 4*, CDSNAc-heparin; *lane 5*, CDSNS-heparin.

whether the putative heparin-binding region (Y^{225}-K-R-K-Q-C-K-P^{232}) of IGFBP-5 is responsible for its binding to heparin. Peptide B (A^{151}-V-K-K-D-R-R-K-K-L-T^{161}), which contains several basic residues but no putative heparin-binding region, was used as a control. ^{125}I-IGF-I was incubated with 100 ng/ml of IGFBP-5 and 10 μg/ml of heparin in the presence or absence of the indicated concentrations of peptide A or peptide B (Fig. 7). Heparin decreased formation of the ^{125}I-IGF-I·IGFBP-5 complex, and complex formation was restored by co-incubation with peptide A. In contrast, peptide B had no effect strongly, suggesting that the region of IGFBP-5 encoded by peptide A was important for IGFBP-5 binding to heparin. To further determine specificity an additional IGFBP-5 peptide was tested for its capacity to alter ^{125}I-IGF-I·IGFBP-5 complex formation. Peptide D, which contains the P^{139}-K-H-T-R-I^{144} sequence, also had no effect (data not shown). In order to rule out the possibility that peptide A directly alters the binding of ^{125}I-IGF-I to IGFBP-5, ^{125}I-IGF-I was incubated with 100 ng/ml of IGFBP-5 and increasing concentrations of peptide A in the absence of heparin, then cross-linking was performed. Peptide A had no effect on ^{125}I-IGF-I binding to IGFBP-5 (data not shown).

We reported previously that IGFBP-5 bound specifically to fibroblast ECM and that the interaction between IGFBP-5 and fibroblast ECM was inhibited by NaCl (7). Heparin is a highly negatively charged molecule composed of repeating disaccharides with carboxyl and sulfate groups (24). Therefore, the sulfate and/or carboxyl groups of heparin may be important for inhibition of IGF-I·IGFBP-5 complex formation. To examine the importance of the sulfate groups, ^{125}I-IGF-I was incubated with 100 ng/ml of IGFBP-5 in the presence or absence of 200 μg/ml of various chemically modified heparins. CDSNAc-heparin inhibited IGF-I·IGFBP-5 complex formation, but its activity was considerably decreased compared with heparin (Fig. 8, *lanes 2* and *4*). The mean reduction in band intensity compared with control in four experiments was 83 ± 3% ($p < 0.001$) for heparin and 16 ± 17% (p, not significant) for CDSNAc heparin. NDSNAc-heparin, which retains *O*-sulfate groups, was most active, but it did not have an inhibitory effect that was equal to unmodified heparin. The mean reduction for the experiments was 52 ± 15% ($p < 0.02$). The activity of CDSNS-heparin was slightly greater than that of CDSNAc-heparin, but was significantly less than NDSNAc-heparin. The mean reduction was 21 ± 17% (p, not significant). These results indicate that *O*-sulfate groups of heparin are important to obtain inhibitory effects of heparin and that *N*-sulfation has only minimal effects on total heparin activity.

We examined whether other negatively charged polysaccharide substances that contain *O*-sulfate groups could alter IGF-I·IGFBP-5 complex formation. Dextran sulfate (M_r <10,000), pentosan polysulfate and fucoidan were tested for activity. ^{125}I-IGF-I was incubated with 100 ng/ml IGFBP-5 in the presence or absence of 100 μg/ml of these polyanionic substances. All three polyanionic compounds inhibited formation of IGF-I·IGFBP-5 complexes (Fig. 9). The reductions in band intensities were 85% for dextran sulfate, 55% for pentosan polysulfate, and 88% for fucoidan.

DISCUSSION

In this paper we report that heparin inhibits formation of the IGF-I·IGFBP-5 complex and that this effect can be mimicked by heparin-like compounds that contain *O*-sulfated saccharide chains. Heparin's activity was detectable, even if the IGF-I·IGFBP-5 complex had been preformed and occurred at heparin concentrations as low as 1.0 μg/ml. The effect occurred rapidly, since complete dissolution of preformed complexes was detectable 15 min after the addition of heparin. The effect appeared to be specific, since the formation of complexes between radiolabeled IGF-I and IGFBP-1, IGFBP-2, or IGFBP-4 could not be inhibited by heparin thus proving that heparin was not nonspecifically interfering with the cross-linking reaction.

Heparin inhibited the binding of IGF-I to IGFBP-5 by decreasing the affinity of IGFBP-5. We hypothesize that binding of heparin to IGFBP-5 leads to a conformational change that results in a reduction of its affinity for IGF-I and that the binding sites within IGFBP-5 for heparin and IGF-I are different. In support of this hypothesis is the experimental observation that heparin resulted in a 17-fold reduction in the affinity of IGFBP-5 for IGF-I. Furthermore, peptide A, which contains a putative heparin binding domain of IGFBP-5, inhibited the effect of heparin on IGF-I binding to IGFBP-5. However peptide A did not directly inhibit ^{125}I-IGF-I binding to IGFBP-5. This is direct evidence that the heparin binding domain of IGFBP-5 is not the same as the IGF-I binding site. It is still possible that IGFBP-5 might bind heparin through two sites one of which contains the IGF-I binding site and the other which does not. However, when we tested a synthetic peptide that contains the only other known potential heparin binding site within IGFBP-5 (*e.g.* the BBXB sequence), it had no effect on heparin inhibition of IGF-I binding, suggesting that only one binding

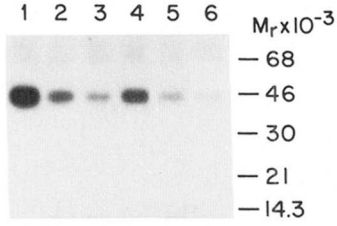

FIG. 9. **Inhibitory effects of dextran sulfate, pentosan polysulfate, and fucoidan on formation of the IGF-I·IGFBP-5 complex.** ^{125}I-IGF-I (30,000 cpm/tube) and IGFBP-5, 100 ng/ml, were added to 100 μl of EMEM supplemented with 20 mM HEPES, pH 7.3, and incubated in the presence or absence of 100 μg/ml of each sulfated polysaccharide at room temperature for 1 h. Samples were then cross-linked with DSS, and SDS-PAGE was performed using a 12.5% gel as described under "Experimental Procedures." *Lane 1*, control; *lane 2*, heparin; *lane 3*, dextran sulfate; *lane 4*, pentosan polysulfate; *lane 5*, fucoidan; *lane 6*, unlabeled IGF-I, 100 ng/ml.

site for heparin was present in IGFBP-5. IGFBP-3, like IGFBP-5, contains the peptide A sequence, and this sequence appears to function as a heparin binding domain. The affinity of IGFBP-3 for IGF-I is also directly lowered by heparin binding but heparin concentrations as high as 500 μg/ml were required to detect a reduction in ^{125}IGF-I·IGFBP-3 band intensity that was equivalent to the reduction in ^{125}I-IGF-I·IGFBP-5 band intensity with 10 μg/ml heparin. In spite of its ability to bind weakly to heparin, IGFBP-4 did not show a change in ^{125}I-IGF-I·IGFBP-4 band intensity even when 500 μg/ml of heparin was added indicating that it did not undergo an affinity shift. IGFBP-4 does not contain the peptide A sequence suggesting that this sequence may be involved in the affinity shift that occurs with heparin binding.

Proteoglycans often bind other proteins by forming a bond between the glycosaminoglycans and basic amino acid sequences, suggesting that the basic amino acid groups in IGFBP-5 might play an important role in its attachment to heparin. Peptide A, which contains a putative heparin domain from positions 225 to 232, completely prevented the inhibitory effect of heparin on IGF-I·IGFBP-5 complex formation. In contrast, peptide B, which is also highly basic but does not contain a heparin binding motif, had no effect. This strongly suggests that the effect of peptide A is due to its BBB*XX*B motif and not its basic charge. An additional peptide, peptide D, which contains the BB*X*B motif that is present in many other heparin-binding proteins (13) had no effect, suggesting that the BBB*XX*B motif was required. However, since a 1000-fold excess of peptide A was required to compete for IGFBP-5 binding to heparin, we cannot absolutely exclude that other regions of IGFBP-5 may be involved in the heparin IGFBP-5 interaction.

Heparin-binding proteins such as antithrombin III, apolipoprotein E and apolipoprotein B-100 are believed to form a helical wheel configuration in which the basic residues are clustered asymmetrically on one side of the helical wheel (13). Helical wheel analysis of the 18-amino acid region of IGFBP-5 that flanks the BBB*XX*B motif in peptide A (residues 221–238) also has this characteristic. In contrast helical wheel analysis of the regions of IGFBP-5 contained in peptides B and D shows that they have more symmetric charge distributions. Similarly IGFBP-1 and -2 contain BB*X*B motifs, but helical wheel analysis shows that their basic residues are not clustered on one side of the helical wheel.

Glycosaminoglycans other than heparin also inhibited the IGF-I·IGFBP-5 interaction. The common structural characteristic that is present in heparin, heparan sulfate, and dermatan sulfate, but not in the other GAGs that were tested, is the presence of *O*-sulfate groups linked to iduronic acid at the 2 or 3 carbon positions (25). Of interest is the observation that dermatan sulfate was by far the weakest of these three compounds in inhibiting complex formation. Dermatan sulfate contains fewer *O*-sulfated groups that are linked to the 2 carbon of iduronic acid as compared with heparan sulfate or heparin, suggesting that the number of *O*-sulfate groups that are linked in this manner is also important. The importance of *O*-sulfates is further substantiated by our findings with chemically modified heparins which showed that completely desulfated heparin or completely desulfated, *N*-resulfated heparin had either no activity or very minimal activity. To determine whether iduronic acid was the only type of saccharide that would have activity, compounds with different saccharide units but which contained *O*-sulfate groups were tested. Dextran sulfate, pentosan polysulfate, and fucoidan all had significant inhibitory activity, further indicating that the type of saccharide ring was not important but that *O*-sulfate groups in either the 2 or 3 positions were necessary.

Heparin-like GAGs, such as heparan sulfate and dermatan sulfate, are present on cell surfaces and in ECM. Numerous cross-linking studies have shown that both IGFBP-3 and IGFBP-5 are cell surface associated. However unlike IGFBP-1, which can bind to cell surfaces through a specific interaction between its RGD sequence and an integrin receptor (26), these forms of IGFBPs have no known receptor. Therefore their binding to cell surface or to ECM could be mediated by binding of basic amino acid groups in these IGFBPs to specific proteoglycans on the cell surface or in the ECM. We have reported previously that the affinity of IGFBP-5 for IGF-I is 8-fold lower when it is associated with ECM (7) and that the affinity of IGFBP-3 for IGF-I is lowered 12-fold when it is bound to cell surfaces (27). Likewise Martin *et al.* (28) reported recently that when human fibroblast cells were incubated with heparin, the amount of IGF bound to cell surface-associated IGFBP-3 was markedly decreased. The mechanism by which IGFBP-5 binding to ECM or IGFBP-3 binding to cell surface lowers their affinities for IGF-I is unknown, but our findings suggest that heparin-like molecules cause a direct reduction in affinity probably through a conformational change for IGFBP-5 and possibly for IGFBP-3. Such a conformational change has been proposed for vitronectin association with heparin (13). Likewise exposure of heparin to antithrombin III also results in a major conformational change (29). Our data showing that heparin and IGF-I have different binding sites on IGFBP-5 are in support of this hypothesis. The significance of this affinity shift to IGFBP-5 modulation of IGF-I action is unknown; however, in previous experiments we have shown that when IGFBP-5 is associated with ECM, it is capable of potentiating the effects of IGF-I on cell growth. A simple model would be that reduction in its affinity for IGF-I allows for more sustained release of IGF-I to receptors and that this slow time-dependent release results in potentiation of the cell growth response. As noted previously, modulation of growth factor activity by GAGs has been shown in several *in vitro* test systems (10, 11)

In addition to dissolution of this complex following cell surface or ECM association, heparin-like compounds may also result in changes in IGF-I binding to serum-binding proteins. Addition of heparin to serum results in dissolution of the acid labile subunit·IGFBP-3·IGF-I complex (15, 30). The molecular mechanism by which IGF-I leaves this tightly bound complex and is transported out of the extravascular space is unknown, but the presence of glycosaminoglycans either in serum (15) or on endothelial cell surfaces (16) has been proposed as a mechanism for dissolution of the complex. This might operate through a similar affinity-lowering effect that is dependent upon heparin binding.

In summary the significance of IGFBP-3- and IGFBP-5-binding glycosaminoglycans in modulating IGF-I interaction with cell surface receptors is incompletely defined. These studies provide a format for analyzing mechanisms of how the affinity of the IGF-binding proteins might be altered by heparin or GAG interactions. Since reductions in IGFBP affinity by dephosphorylation or proteolysis have been shown to result in potentiation of IGF-I action in several systems, determination of the effect of this heparin-IGFBP interaction on IGF-I-mediated actions will be important future studies.

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